SELF-EXCHANGE OF SODIUM IN HUMAN LYMPHOCYTES

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ABSTRACT Self-exchanges of Na and K in human lymphocytes were measured by isotopic efflux techniques. In washed cells, K exchanged in a single slow exponential fraction, but the Na exchange had a marked curvature. It was shown that the curvature was not caused by simple bulk-phase diffusion, and it was resolved into three major fractions: fast (F) (half-time, $t_{1/2}$ = 2–4 min), intermediate (I) ($t_{1/2}$ = 12 min), and slow (S) ($t_{1/2}$ = 125 min). Each of these appeared to follow an exponential function. The I fraction contained ~10 mmol Na/kg cells (25–30% of normal cellular Na), was not affected by manipulations that cause lymphocytes to gain Na, and had little or no temperature dependence. The S fraction of Na in normal cells (S_1) contained ~10 mmol Na/kg cells, had only a slight temperature dependence, and the amount and rate of S_1 were independent of external K concentration (K_{ex}). Another slow fraction (S_2) appeared when the cells underwent a net gain of Na in exchange for K, and was characterized by a steep temperature dependence and a peak rate around the transition point (the point at which half of cellular K is replaced by Na) at 0.4 mM K_{ex}. The results are discussed within context of a theory that assigns the exchange of the major part of K in its slow exponential fraction and the Na exchange in S_2 to interactions of these ions with fixed anionic sites, on intracellular macromolecules, which have been shown previously to interact cooperatively in their association with K and Na.

INTRODUCTION

In previous studies of ionic self-exchange in human lymphocytes we detected two fractions of K and at least two fractions of Na (Negendank and Shaller, 1979*b*, 1980*b*). Ionic influx or efflux in the slowest (S) fractions appeared to follow exponential functions. 97% of the normal amount of cellular K (~134 mmol/kg wet cell wt) exchanged with ^{42}K in the slow exponential fraction with a half-time ($t_{1/2}$) of ~200 min. Approximately 25% of the normal amount of cellular Na (9 mmol/kg cells) exchanged with ^{22}Na in the slowest exponential fraction with a $t_{1/2}$ of 120 min. Another 25% of cellular Na exchanged at an intermediate rate with a $t_{1/2}$ of ~12–14 min. The remaining 3% of K (4 mmol/kg cells) and ~50% of Na (18 mmol/kg cells) exchanged rapidly with ^{42}K and ^{22}Na, respectively, with $t_{1/2}$'s of ~2 min. The K and Na that exchanged in these fast (F) fractions also exchanged rapidly in or out of the cell when net influxes or effluxes of these ions occurred when the concentration of K or Na was changed in the external medium (Negendank and Shaller, 1980*a*, 1981, 1982*c*). Moreover, the amounts of K and Na that exchanged in the F fractions bore a nonsaturable relation to the external concentration of these ions (Negendank and Shaller, 1979*b*, 1980*a*, 1981).

From detailed analysis of ^{42}K and ^{22}Na effluxes and influxes we concluded that the F fractions exchange in series with the slower ones and their rates are determined by the permeability of the cell's surface membrane. The amounts of K and Na that exchanged in the F fractions and that were nonsaturable with increased external ion concentration were thought to be the K and Na that are dissolved within ordered cellular water at equilibrium concentrations less that in the external medium. The amounts of K and Na that exchanged in the S fractions and were saturable with increased external ion concentration were thought to be adsorbed onto intracellular macromolecules; self-exchanges of these ions were rate-limited by adsorption onto and desorption from fixed anionic charges. These concepts were recently reviewed (Negendank, 1982).

A number of experimental conditions cause human lymphocytes to lose K and replace it with Na. These include incubation in 0 K_{ex}, metabolic inhibition, treatment with ouabain, and incubation at 0°C. In studies on the temperature dependence of self-exchanges of K and Na and on the temperature dependence of ouabain effect on them, we found the S fraction of Na exchange did not have a strong temperature dependence when the cells contained a normal amount of Na, but acquired a strong temperature dependence whenever the cells gained Na. In contrast, K self-exchange had a strong temperature dependence when the cells contained a normal amount of K (Negendank and Shaller, 1982*c*). This observation led us to suggest that there are two potential S fractions of Na exchange. One, designated S_1, is the exchange of an adsorbed fraction of

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Na that is present within the normal cell. The second, S_2, appears when K is replaced by Na and its properties are determined by a large, separate set of adsorption sites that normally adsorb K.

A number of questions are raised by these results and the concepts derived from them: (*a*) Is the separation of the Na self-exchange into a minimum of three exponential processes justified, or is all or part of the curvature of Na efflux due to simple bulk-phase diffusion of Na within the cell or to a major inhomogeneity in the cell population? (*b*) What are the properties of the apparent intermediate (I) fraction of Na self-exchange, and what are its possible sources? (*c*) Is there independent evidence for the separation of the S_1 and S_2 fractions of Na self-exchange? (*d*) Because S_2 appears only when the cells gain Na to replace K, does Na exchange in S_2 have properties similar to those of K exchange in its normal S fraction?

To investigate these questions we studied ^{22}Na and ^{42}K effluxes under a variety of conditions and included studies using a washing technique that enhances analysis of the I and S fractions of Na efflux by removing extra-cellular ^{22}Na and the F fraction of cellular ^{22}Na.

METHODS

Lymphocytes for each experiment were prepared from 400 ml of fresh peripheral blood from healthy adults. They were separated from blood by a Hypaque-Ficoll gradient. Platelets were removed by a series of washes in large volumes of solution and then centrifuged at 200 *g* for 20 min as described previously (Negendank and Shaller, 1979*a*). Cells were incubated under sterile conditions in flat-bottom, 250-ml plastic bottles that contained 50–100 ml of cell suspension. The solution used to separate the cells from the blood contained 145 mM Na, 5.5 mM K, 136 mM Cl, 1.3 mM Ca, 1.0 mM Mg, 14.3 mM HCO_3, 0.34 mM HPO_4, 1.3 mM H_2PO_4, and 5.6 mM glucose. The pH was 7.4 and stable in sealed containers. The incubation medium was identical, except it contained 10% autologous serum. Cells were incubated without agitation at 37°C for 24–48 h before ^{22}Na efflux was studied to ensure that the net cellular contents of ions and water were time independent (except in the studies with ATP-depletion). Experimental subjects included control cells at 37°C, cells at lower temperatures, cells treated with ouabain (5×10^{-6} M) (Sigma Chemical Co., St. Louis, MO), cells at various external K concentrations (K_{ex}), and cells depleted of ATP by metabolic inhibition caused by iodoacetate (IAA) and N_2. In previous studies, we documented by flaming the absence of a net gain or loss of Na or K under most of these conditions. Because the experimental conditions reported here are identical to those used previously, we did not repeat determinations of the net ionic contents of the cells. The cellular Na and K contents are summarized in Table I, and details are available in the references cited there.

Cells were preloaded with ^{22}Na or ^{42}K (New England Nuclear, Boston, MA) by incubating them in medium containing the isotope for the last 20–24 h of the pre-equilibration procedure. This ensures that ^{22}Na or ^{42}K reaches equilibrium with total cellular Na or K (Negendank and Shaller, 1980*b*). We assume therefore that there is uniform distribution of isotope throughout the various compartments of the cell and that the tracer isotope behaves the same as the unlabeled ion.

To study ^{22}Na or ^{42}K efflux, we transferred the cells to nonlabeled medium and followed the appearance of isotope. In some experiments, cells were separated from the labeled preloading medium without washing, as described previously (Negendank and Shaller, 1979*b*, 1980*b*). In other experiments, cells were washed after preloading with isotope. In the latter case, the cell suspension was transferred to a 50 ml polypropylene tube, packed gently at 250 *g* for 10 min, resuspended in 15 ml of nonlabeled medium, and packed at 1,700 *g* for 2 min. The wash was repeated and the cells resuspended in nonlabeled medium to determine the ^{22}Na or ^{42}K efflux. The entire washing procedure took 6–7 min, from the time when the cells were removed from labeled preloading medium to when they were immersed in nonlabeled medium to begin the efflux study. This medium was otherwise identical to the preloading medium in composition and temperature.

TABLE I
APPROXIMATE CELLULAR CONTENTS OF IONS AND WATER UNDER THE CONDITIONS STUDIED

Condition	Time	Temperature	Water	K	Na
	h	°C	*% wet wt*	*mmol/kg wet wt*	
Control	48	37	77.8	139	28.9
ATP-depleted (IAA, N_2)	3	37	73.6	94	82
Ouabain (5×10^{-6} M)	48	37	78.6	15.7	139
0°C	48	0	77.5	35.9	128

Data are means from previous studies under the same conditions used for the isotope exchange studies reported here. Control and Ouabain (Negendank, 1984); ATP-depleted (Negendank and Shaller, 1982*a*); 0°C (Negendank and Shaller, 1982*c*).

RESULTS

Comparison of the Self-Exchanges of K and Na

Efflux of ^{42}K from cells pre-equilibrated at 37°C is shown in Fig. 1. In *a–c*, cells were washed twice in nonlabeled medium before beginning the efflux study, and in *d–e*, they were not washed. Washing removed most of the ^{42}K-containing preloading medium as well as the very fast fraction of exchange of cellular K documented in previous studies (Negendank and Shaller, 1979*b*, 1982*b*). The majority of K (which is ~97% of total cellular K) exchanged in a single exponential fraction for at least 3 h. Efflux of ^{22}Na is shown in Fig. 2, where cells were washed twice in nonlabeled medium before beginning the efflux study (*a–c*), and where they were not washed (*d–e*). In contrast to the efflux of ^{42}K (Fig. 1), the efflux of ^{22}Na has a marked curvature in the first 30–60 min. This is followed by an apparent exponential exchange that is present for at least 3 h. The different behavior of K and Na is also shown in Fig. 3, in which ^{42}K and ^{22}Na effluxes were determined simultaneously.

The exponential nature of the self-exchange of the major part of cellular K is compatible with a rate-limiting step determined either by a barrier effect (such as a membrane separating two dilute solutions) or by a reversible chemical reaction (such as an adsorption-desorption process within the cell) (Sheppard, 1948). On the other hand, it is conceivable that the curvature of the Na self-exchange represents a discrete series of two or three exponential functions, multiple exponential or nonexponential fractions merging together, or a simple bulk-phase diffusional pro-

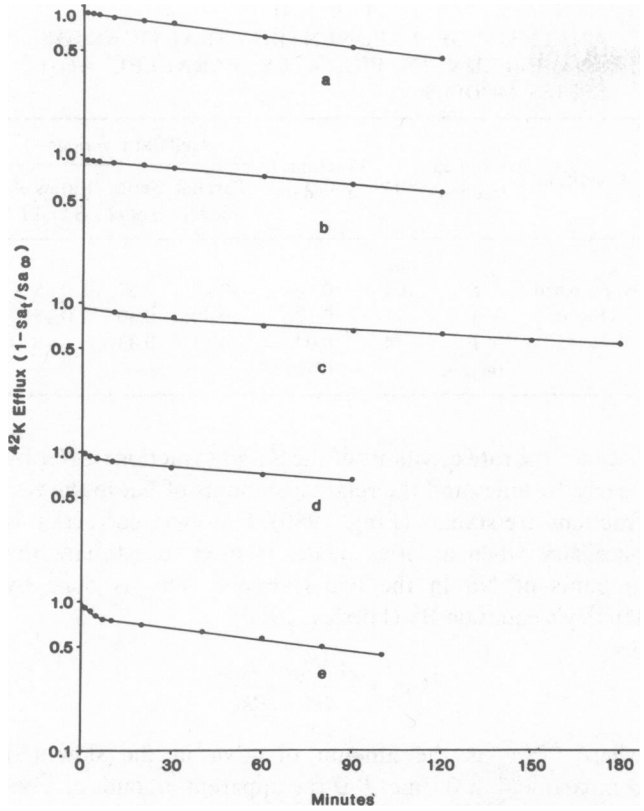

FIGURE 1 K self-exchange determined by ^{42}K efflux. Cells in *a-c* were washed twice after they were preloaded with ^{42}K for 24 h. Cells in *d* and *e* were not washed. Data are expressed as the rate of rise of specific activity (sa_t) of ^{42}K in the medium, with sa_∞ taken as that of the suspension. Cells were at a temperature of 37°C.

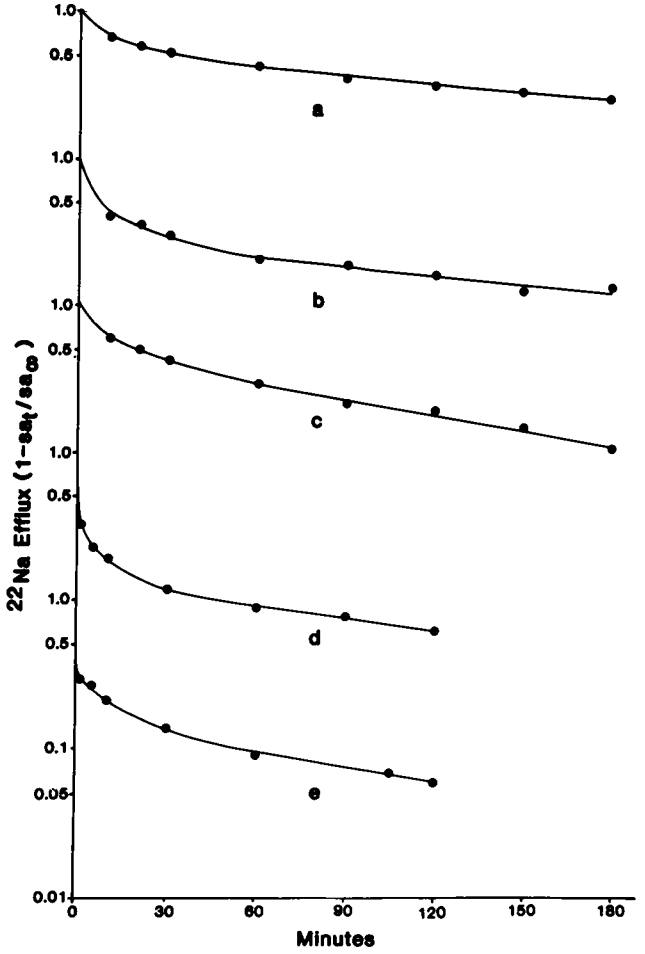

FIGURE 2 Na self-exchange determined by ^{22}Na efflux. Cells in *a–c* were washed twice after they were preloaded with ^{42}K for 24 h. Cells in *d* and *e* were not washed. Data are expressed as the rate of rise of sa_t of ^{22}Na in the medium, with sa_∞ taken as that of the suspension. Cells in *a–d* were at 37°C, and in *e* at 20°C.

cess. Analysis of these possibilities will occupy the next four sections of Results.

Simple Compartmental Analysis of ^{22}Na Efflux

This analysis is appropriate for steady state isotopic exchange in which the driving force for the diffusion is simply the specific activity of the solute, and in which diffusion is rate-limited either by discrete barriers between dilute solutions or by a chemical reaction or adsorption-desorption process (Sheppard, 1948; Solomon, 1960). In either case, isotope exchange follows first-order kinetics and is expected to fit an exponential function. The method of analysis is illustrated in Fig. 4, where it is applied to a large series of studies that were published previously (Negendank and Shaller, 1982*c*; Fig. 2, 37°C), which provide sufficient early time points for analysis. The slowest apparent exponential fraction was extrapolated to the ordinate. This was subtracted from the total and a second, intermediate exponential fraction drawn and extrapolated to the ordinate. This was subtracted from the remaining total to yield the fastest fraction. Thus, the data could be approximated by a minimum of three exponential functions.

The three fractions in Fig. 4 account for ~30% of the total ^{22}Na in the system; the remainder exchanges instantly and its source is the labeled preloading medium that is carried over with the cells (Negendank and Shaller, 1979*b*, 1980*b*). Each exponential fraction, *i*, is characterized by a rate constant, k_i, in the equation

$$^{22}Na_{i_t}/^{22}Na_{i_\infty} = e^{-k_i t}, \quad (1)$$

where the subscript t is the time of sampling. The $t_{1/2}$'s of the S, I, and F fractions are 104, 11, and 4 min, respectively.

The I and S fractions of ^{22}Na efflux either could occur in parallel or in series with one another. Parallel exchange would occur if, for example, they existed in different cells. Series exchange would occur if, for example, the I fraction were limited by diffusion across the surface and the S fraction were limited by diffusion out of a subcellular compartment. If the fractions exchanged in parallel, extrapolation of the exponentials to the ordinate would give

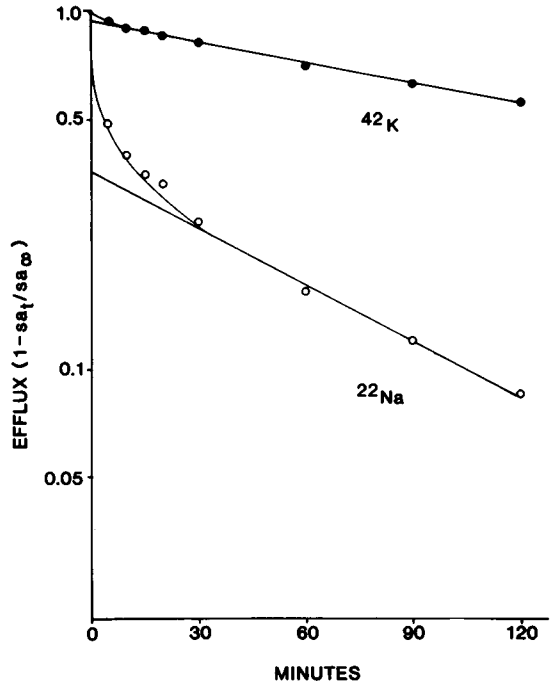

FIGURE 3 Simultaneous ^{42}K and ^{22}Na effluxes from cells pre-equilibrated for 48 h in 0.4 mM K_{ex} and 149.6 mM Na_{ex}. The data are presented as means of two separate experiments.

reasonably accurate estimates of the relative amounts of Na in these fractions and their rate constants. If they exchanged in series, extrapolation to the ordinate would underestimate the amount of Na in the slow fractions and overestimate its rate constant. The extent to which these estimates deviate from their actual values depends on the relative amounts of Na in the two fractions and the relative sizes of their rate constants. The rate constants of the extrapolated curves are within 1–2% of their actual values

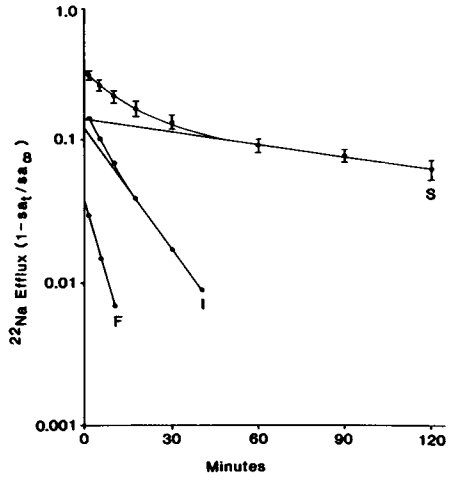

FIGURE 4 Na self-exchange determined by ^{22}Na efflux. Cells were incubated under control conditions (5.5 mM K_{ex}, 145 mM Na_{ex}, 37°C) and were not washed before beginning the efflux study. Data are expressed as in Figs. 1 and 2. Means ± SEM of 12 experiments.

TABLE II
ANALYSIS OF EXPONENTIAL FRACTIONS OF ^{22}Na EFFLUX IN FIG. 4 BY PARALLEL AND SERIES MODELS

Condition	Assigned fraction	$t_{1/2}$	Fractions in raw data	Cellular fractions		
				Parallel model	Series model	Ratios of S and I
		min				
Nonwashed (Fig. 4)	S	104	0.14	0.47	0.37	0.55
	I	11	0.12	0.40	0.30	0.45
	F	4	0.04	0.13	0.33	—
	Instant	—	0.70	—	—	—

because the rate constants of the S and I fractions differ by nearly 10 times and the relative amounts of Na in the two fractions are similar (Ling, 1980). However, correction is necessary when a series model is used to estimate the amounts of Na in the two fractions. This is done by Huxley's equation B8 (Huxley, 1960):

$$^{22}Na_{s_0} = \frac{AB(k_I - k_S)^2}{Ak_I^2 + Bk_S^2}, \quad (2)$$

where $^{22}Na_{s_0}$ is the amount of ^{22}Na in the slow (S) compartment at 0 time, B is the apparent amount of ^{22}Na in S determined by extrapolation, A is the apparent amount of ^{22}Na in the intermediate (I) compartment determined by extrapolation, and k_I and k_S are the apparent rate constants of the I and S fractions. A similar analysis was applied to separate the I and F fractions in Fig. 4.

Using this simple compartmental analysis, the relative sizes of the Na fractions obtained from parallel and series models are shown in Table II.

Simple Bulk-Phase Diffusional Analysis of Na Self-Exchange

Although the separateness of the F fraction of Na exchange was documented independently by its ability to undergo rapid net exchanges (Negendank and Shaller, 1979*b*, 1980*a*, 1981, 1982*c*), it is conceivable that part or all of the curve of Na self-exchange is a consequence of simple diffusion within the bulk interior of the cell. This may be the case for the entire (FIS) curve, or for a part of the curve (i.e., IS). The data in Fig. 4 are replotted in Fig. 5 as the curves labeled FIS and IS, along with the slowest exponential fraction, S. A series of simple bulk-phase diffusion curves for the diffusion of Na within the water of a sphere, which is roughly the size of the lymphocyte, are also plotted using different assumed values for the diffusion coefficient, D (in cm^2/s), as calculated by Ling et al. (1967) from equations of Dünwald and Wagner (1934):

$$\frac{\partial^{22}Na}{\partial t} = D\left(\frac{\partial^2\,{}^{22}Na}{\partial r^2} + \frac{2}{r}\frac{\partial^{22}Na}{\partial r}\right), \quad (3)$$

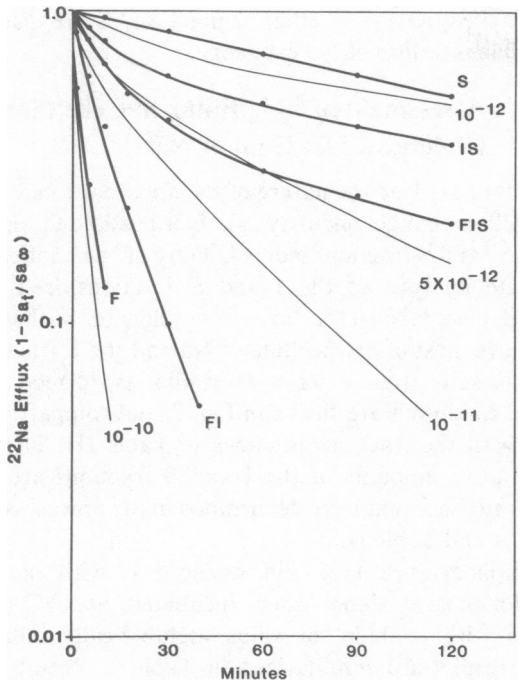

FIGURE 5 Semilogarithmic plot of fractions of ^{22}Na efflux and of simple bulk-phase diffusion curves. The apparent fractions are derived from the experiments in Fig. 4. FIS is the whole curve in Fig. 4. Diffusion coefficients (cm^2/s) are derived for a sphere 10 μm in diameter, using Eq. 3.

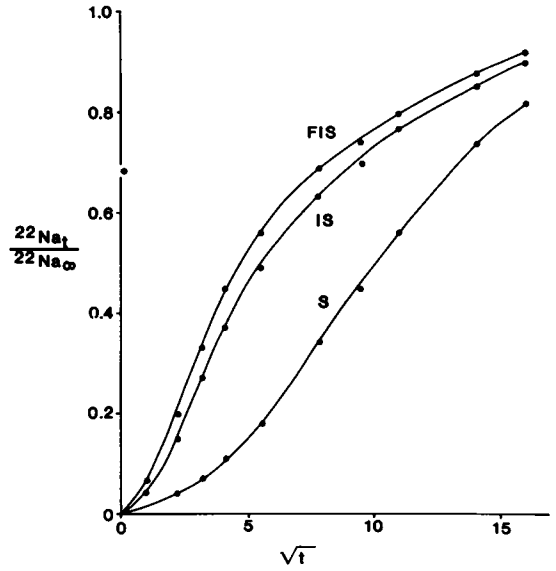

FIGURE 6 $\sqrt{t}$ plot of the data in Fig. 5.

where r is the radius (5×10^{-4} cm). For efflux of ^{22}Na from a cell preequilibrated in labeled solution and washed in a solution containing negligible isotope, note that at longer times the diffusion curves approach exponentials approximated by the equation:

$$\ln\left(1 - \frac{^{22}Na_t}{^{22}Na_\infty}\right) = A - B\left(\frac{Dt}{r^2}\right), \quad (4)$$

where A and B are constants, and the ratio $^{22}Na_t/^{22}Na_\infty$ refers to the appearance of ^{22}Na in the external medium (Dünwald and Wagner, 1934; Crank, 1956; Ling et al., 1967).

Discrimination between simple bulk-phase diffusion and an exponential or complex diffusional process may be obtained by plotting fractional exchange of Na as a function of $\sqrt{t}$(Crank, 1956). In this plot, simple diffusion rises steeply at early times, whereas an exchange process of first-order kinetics (barrier, reaction, or adsorption-desorption) has a sigmoidal relation to $\sqrt{t}$. The FIS curve and the IS fraction from Figs. 4 and 5 were plotted in Fig. 6 by using this method; the exponential S fraction is shown for comparison.

The analyses in Figs. 5 and 6 indicate the following: (*a*) The entire FIS curve is not described by simple bulk-phase diffusion. (*b*) The IS curve also is not described by simple diffusion, as shown by the $\sqrt{t}$ plot. Moreover, if simple diffusion caused the efflux of ^{22}Na in IS, the diffusion coefficient, D, would be of the order of magnitude of 10^{-12} cm^2/s (Fig. 5). This is seven orders of magnitude smaller than the coefficient for the diffusion of Na in a dilute solution ($D \sim 10^{-5}$ cm^2/s; Wang, 1952). It is unlikely that this value could characterize diffusion of Na within cellular water, which itself is likely to have a diffusion coefficient within an order of magnitude of that of water in a dilute solution at 25–37°C (Ling et al., 1967; Hazlewood, 1979; Trantham et al., 1984). (*c*) This analysis does not permit a conclusion regarding the nature of the F fraction, i.e., whether or not it is first order. In previous studies, it was concluded that this fraction is determined by the permeability of the surface membrane (Negendank and Shaller, 1979*b*, 1980*b*); hence, it would be expected to follow first-order kinetics. The analysis in Fig. 5 suggests that Na exchanges at a rate that corresponds to a diffusion coefficient on the order of 10^{-10} cm^2/s. This implies that passage of Na through the surface membrane occurs at a rate that is five orders of magnitude slower than free diffusion in cellular water or in the external solution. These statements also apply to the FI fraction of Na, which has a diffusion coefficient of $\sim 3 \times 10^{-10}$ cm^2/s (Fig. 5).

Analysis of Exchanges of Na and K as Simultaneous Diffusion and Reversible Adsorption

This analysis is appropriate for a model in which there is diffusion of an ion between cellular and external water and either reversible adsorption of the ion onto intracellular macromolecules or entry and exit of the ion to and from a membrane-limited subcellular compartment. The form of the exchange curve varies depending upon the relative amounts of ion in cell water and adsorbed, the relative sizes

of the rate constants of adsorption and desorption, the diffusion coefficient, and the time scale of the experiment. Crank (1956, Chapter 8) has shown that if the diffusion rate is more than 10^3 times faster than adsorption or desorption, then the diffusion profile is dominated by the first-order process, whereas if the rates are comparable, the profile is essentially that of simple bulk-phase diffusion. Clearly, exchanges of K and Na in lymphocytes (Figs. 1–5) fall between these limits.

Diffusion from a sphere of radius r is described by the equation:

$$\frac{\partial(M_{f_0} - M_f)}{\partial t} = D\left[\frac{\partial^2(M_{f_0} - M_f)}{\partial r^2} - \frac{2}{r}\frac{\partial(M_{f_0} - M_f)}{\partial r}\right] - \frac{\partial(M_{ad_0} - M_{ad})}{\partial t}, \quad (5)$$

where the ion M that is free in cell water is designated M_f and the adsorbed ion is designated M_{ad}, and where $\partial M_{ad}/\partial t = \lambda M_f - \mu M_{ad}$, in which λ and μ are the rate constants of adsorption and desorption, respectively. The initial conditions are $M_f = M_{f_0}$, $M_{ad} = M_{ad_0}$, $t = 0$, and because the system begins at equilibrium, $\lambda M_{f_0} - \mu M_{ad_0} = 0$.

This approach was used in the analysis of exchanges of K and Na in the dog carotid artery (Jones and Karreman, 1969). In our studies in lymphocytes, however, the choice of D is complicated because movement of K and Na across the surface of the lymphocyte is slowed relative to free diffusion, by ~five orders of magnitude, as shown by the F curve in Fig. 5. The cells are so small ($r = \sim 5 \times 10^{-4}$ cm) that diffusion with $D = 10^{-10}$ cm^2/s and $t_{1/2}$ ~2 min is about the fastest that can be detected in our experiments. Therefore, diffusion of Na in the external solution and within cell water are not detectable, and for the F fraction, it is not possible to distinguish between simple diffusion and a first-order process (as indicated in Fig. 5) because the curvature is slight or nonexistent. Therefore, in the case of K, where there are only two clearly separable fractions, F and S (e.g., Fig. 1, *d* and *e*; Negendank and Shaller, 1979*b*), this analysis reduces to that of two first-order processes in series, which is equivalent to the simple compartmental analysis illustrated for Na in Fig. 4.

In the case of Na, however, one may question whether the FI fraction is a simple diffusional one in series with S_1, in accord with Eq. 5. This seems unlikely, for reasons similar to those mentioned above regarding F alone: (*a*) D, which would be $\sim 3 \times 10^{-10}$ cm^2/s (Fig. 5), is five orders of magnitude smaller than the coefficient for free diffusion in a dilute solution, and (*b*) evidence mentioned in the Introduction strongly suggests that F and I can be manipulated separately.

Therefore, although it is obvious that the data can be fitted with Eq. 5 using an appropriate choice of parameters, this analysis is insensitive to the range of values of D that fits the F and FI Na fractions, and it does not help answer the question whether or not I and S are distinctly separable fractions of Na exchange.

Assessment of ^{22}Na Efflux in Cells that Undergo a Net Gain of Na

To further analyze the nature of the apparent I part of the ^{22}Na self-exchange, one may ask: Is it possible to manipulate the I and S fractions independently of one another? To facilitate analysis of the I and S fractions, cells were washed in nonlabeled solution after preloading with isotope to remove most of extracellular ^{22}Na and the F fraction of ^{22}Na. Results from a series of studies performed under control conditions are shown in Fig. 7, and compartmental analysis of the fractions is shown in Table III. The rates and relative amounts of the I and S fractions are quite similar to those that were determined in the unwashed cells in Fig. 4 and Table II.

Lymphocytes lost K and replaced it with Na in a mole-for-mole fashion when incubated at 0°C, when treated with ouabain, or when metabolically inhibited. These results are summarized in Table I. Results from ^{22}Na efflux studies performed under these conditions suggest that when the cells gain Na, the gained Na exchanges within the slowest exponential fraction (Negendank and Shaller, 1982*a*, 1982*c*). However, there are too few data points in these experiments to reliably separate the I and S fractions. Therefore, we studied ^{22}Na efflux under these same conditions using the washing technique, and the results are shown in Figs. 8 and 9. When the cells gained Na by treatment with ouabain (Fig. 8 *a*), by metabolic inhibition (Fig. 8 *b*), or at 0°C. (Fig. 9), there was a reduction in the curvature of ^{22}Na efflux. This reduction shows that a majority of the gained Na exchanged in the S fraction, not in the I fraction.

These results suggest the following. (*a*) The I and S fractions are distinctly separate ones, as we assumed in the

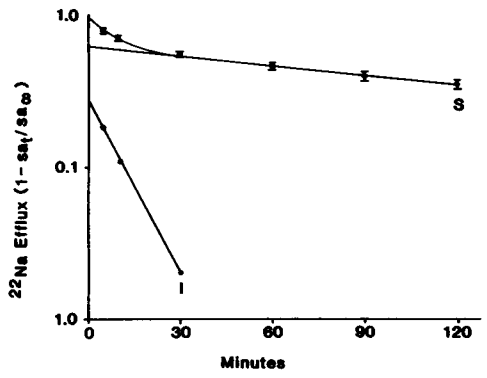

FIGURE 7 Na self-exchange determined by ^{22}Na efflux. Cells were incubated under control conditions (5.5 mM K_{ex}, 145 mM Na_{ex}, 37°C) as in Fig. 4, but they were washed in nonlabeled medium before beginning the efflux study. Data are means ± SEM of five separate experiments (i.e., experiments with cells from five separate subjects).

TABLE III

ANALYSIS OF EXPONENTIAL FRACTIONS OF ^{22}Na EFFLUX IN FIG. 7 BY PARALLEL AND SERIES MODELS

Condition	Assigned fraction	$t_{1/2}$	Fractions in raw data	Cellular fractions		
				Parallel model	Series model	Ratios of S and I
		min				
Washed (Fig. 7)	S	130	0.63	0.67	0.56	0.56
	I	8	0.30	0.33	0.44	0.44
	Instant	—	0.07	—	—	—

simple exponential analysis in Figs. 4 and 7 and Tables II and III. (*b*) If the curvature of ^{22}Na efflux is caused by inhomogeneity of the cell population, then the population(s) bearing the I fraction(s) would appear not to be sensitive to the effects of ouabain, metabolic inhibition, or low temperature.

The rate of Na exchange in the S fraction was affected differently by the different means used to cause lymphocytes to gain Na. Ouabain (Fig. 8 *a*) had little, if any,

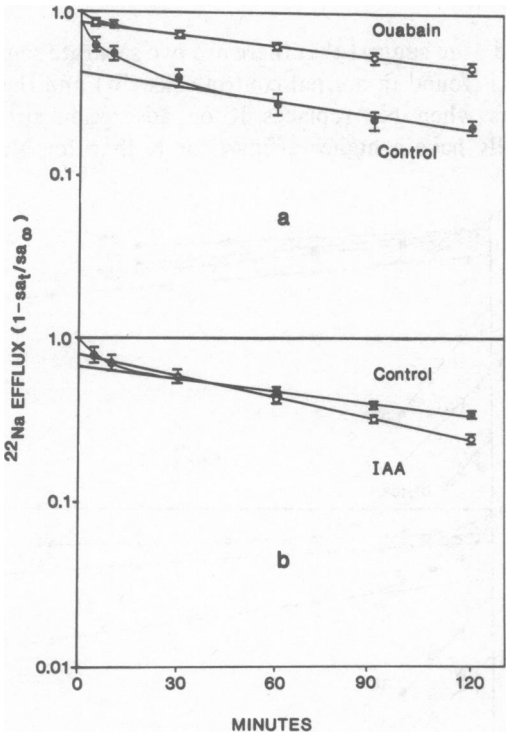

FIGURE 8 ^{22}Na efflux from cells that have undergone a net gain of Na. Cells were studied as in Fig. 7 and were washed twice after preloading with ^{22}Na. (*a*) Cells pre-equilibrated for 48 h, and preloaded with ^{22}Na for 24 h, with and without 5×10^{-6} M ouabain. (*b*) Cells preloaded 24 h with ^{22}Na and then incubated 3 h with and without IAA, N_2 (still in ^{22}Na) before ^{22}Na efflux was measured. Means ± SEM of three separate experiments under each condition using control and treated cells from each subject simultaneously. Approximate net ionic contents of these cells are shown in Table I.

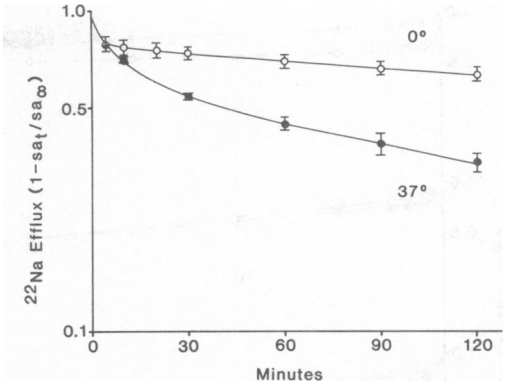

FIGURE 9 Comparison of ^{22}Na efflux from cells equilibrated at 37° and 0°C. Those at 37°C contained ~29 mmol Na/kg cell wet wt, and those at 0°C contained ~125 mmoles Na/kg wet cell wt (Table I). Data are presented as means ± SEM of four separate experiments at 0°C and six experiments at 37°C.

effect, whereas low temperature (0°C) slowed the rate down (Fig. 9). With metabolic inhibition (Fig. 8 *b*), the cells underwent a net gain of Na (Table I; Negendank and Shaller, 1982*a*), yet their ^{22}Na efflux was faster than in controls. A similar effect occurred when lymphocytes were first placed into medium containing 0 K_{ex} (Fig. 10). Because these cells in IAA or in 0 K_{ex} are undergoing a net gain of Na, the unchanged or increased ^{22}Na efflux implies that there is also an increase in the amount of Na moving out of the cell.

Temperature Dependence of the I and S Fractions of ^{22}Na Efflux

A number of individual experiments in cells pre-equilibrated at different temperatures are shown for cells incubated in 5.4 mM K_{ex} (Fig. 11) and for cells incubated in ~0 K_{ex} (Fig. 12). The position of the curves with respect to the

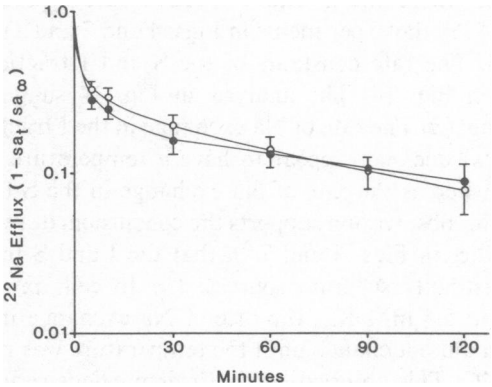

FIGURE 10 ^{22}Na efflux from cells measured after exposure to medium containing 0 mM K_{ex} or 5.5 mM K_{ex}. Cells were pre-equilibrated for 24–48 h in control medium containing 5.5 mM K_{ex}, separated from the preloading medium without washing, and immersed in either 5.5 mM K_{ex} (●) or 0 mM K_{ex} (○) to determine the ^{22}Na efflux. Data are presented as means ± SEM of four separate experiments.

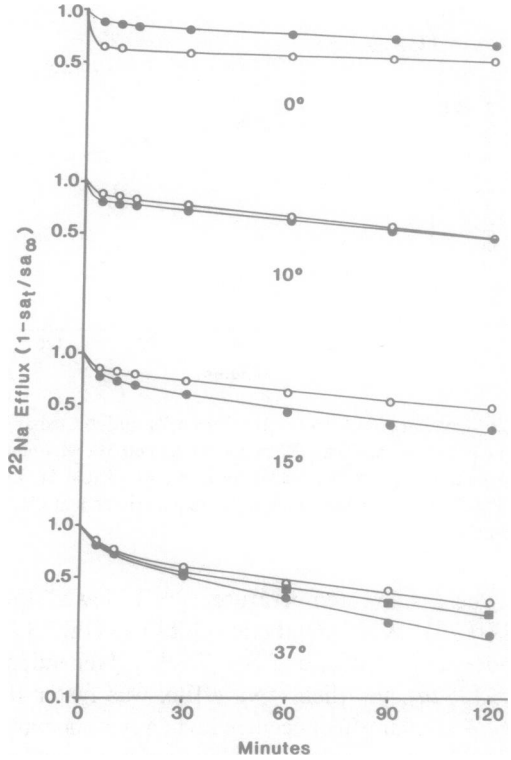

FIGURE 11 Temperature dependence of ^{22}Na efflux in cells pre-equilibrated at 5.4 mM K_{ex}. Cells were pre-equilibrated, and ^{22}Na efflux was studied at the temperatures indicated. Single experiments were chosen to show the extremes noted in the entire series of studies.

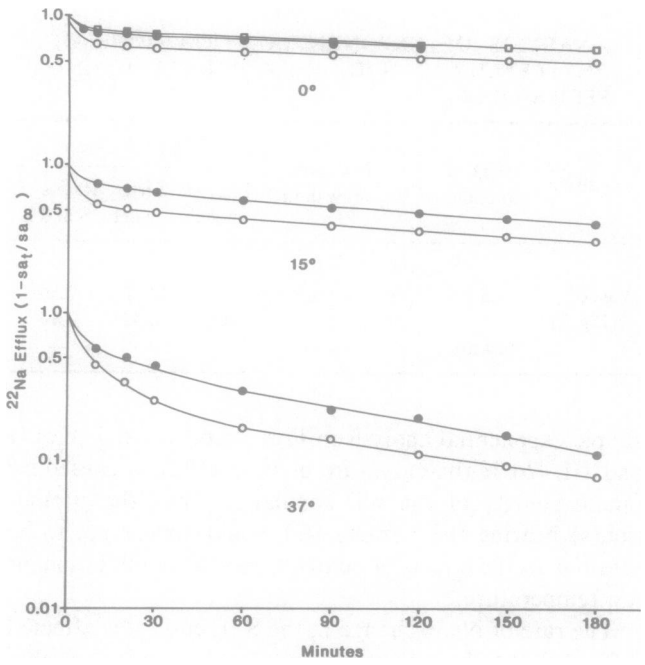

FIGURE 12 Temperature dependence of ^{22}Na efflux in cells pre-equilibrated at 0 mM K_{ex}. Details are the same as in Fig. 11.

ordinate is determined as much or more by variables, such as the degree of washing away of labeled preloading medium, than by differences in the Na exchange rate of the S fraction at each temperature. Therefore, it is misleading to present the data as a means ± SE, and to analyze the results we use the averages of all experiments done at each temperature. These results are summarized in Fig. 13, where the S and I fractions are separated as described in the experiments in Figs. 4 and 7 and Tables II and III. The rate constants of the S and I fractions are shown in Fig. 14. The analysis in Fig. 14 suggests the following: (*a*) The rate of Na exchange in the I fraction (or fractions) does not appear to have a temperature dependence, whereas the rate of Na exchange in the S fraction does. This observation supports the conclusion, drawn from the studies in Figs. 4 and 7–9, that the I and S fractions have distinctly separate sources. (*b*) In cells pre-equilibrated in 5.4 mM K_{ex}, the rate of Na exchange in the S fraction did not change until the temperature was reduced to <15°C. This observation confirms previous results and coincides with the finding that the net gain of Na in exchange for K does not occur until temperature is reduced to <15°C (Negendank and Shaller, 1980*a*, 1982*c*). (*c*) The acquisition of a steep temperature dependence, which occurred only when the cells were incubated at a temperature low enough to cause some cellular K to be replaced by Na, led us to suggest that there are two separate sources of S: one is found in normal control cells (S_1) and the other appears when Na replaces K on adsorption sites that normally have a higher affinity for K than for Na (S_2).

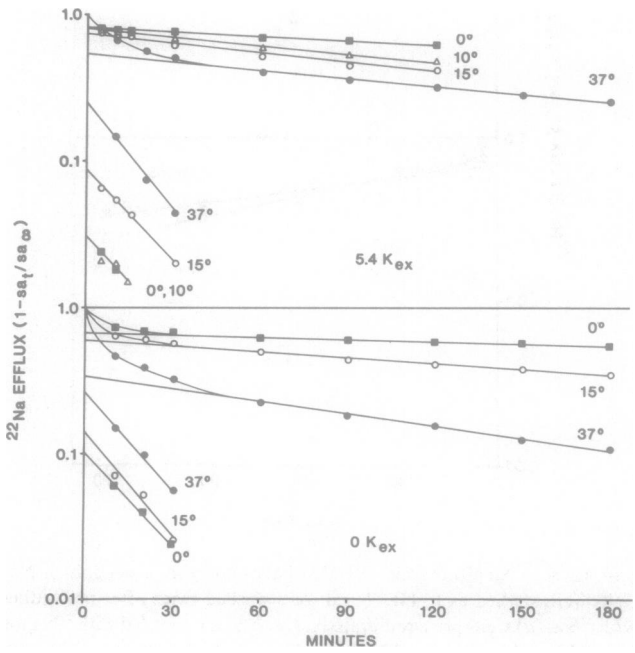

FIGURE 13 Temperature dependence of ^{22}Na efflux. Cells at the top were pre-equilibrated in 5.4 mM K_{ex} (as in Fig. 11), and cells at the bottom in ~0 mM K_{ex} (as in Fig. 12), at the indicated temperatures. Data are means of two or three separate sets of experiments under each condition. Exponential analysis was done as shown in Figs. 4 and 7.

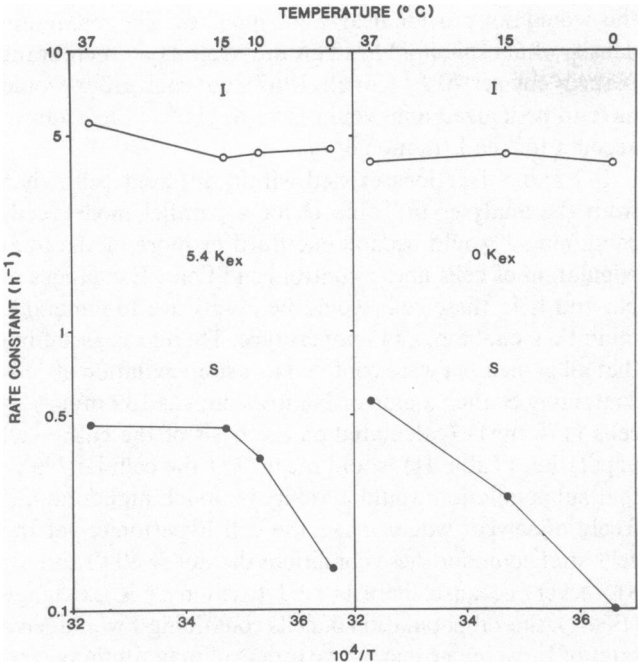

FIGURE 14 Arrhenius plots of ^{22}Na efflux. Rate constants (h^{-1}) labeled S were derived from the slowest exponential fractions of the data in Fig. 13, and rate constants labeled I were derived from the faster exponential fractions shown in Fig. 13. The activation energies (E_a) for Na self-exchange were calculated from the equation $-E_a/R = \Delta \ln k/\Delta T^{-1}$, where k is the rate constant, R is the Boltzmann factor, and T is °K. E_a of the S fraction is 1.2 kcal/mol between 37° and 15°C and 11.0 kcal/mol between 15° and 0°C. E_a of the I fraction is at most 1.5 kcal/mol between 37° and 0°C. These values may be compared with E_a of self-diffusion of Na in a dilute NaCl solution at 4.4 kcal/mol (Wang, 1952).

When cells are induced to gain Na at all temperatures by using ouabain, they acquire a steeper temperature dependence of ^{22}Na exchange over the entire temperature range (Negendank and Shaller, 1982*c*). The experiments summarized on the right side of Fig. 14 show that cells that undergo a net gain of Na and loss of K at 0 K_{ex} also acquire a steeper temperature dependence of ^{22}Na exchange over the entire range.

^{22}Na Efflux from Cells Pre-equilibrated in Different Concentrations of K_{ex}

The results of these studies suggest a way to examine the K_{ex} dependence of Na exchanges in all three postulated fractions (I, S_1, and S_2). Because, at 37°C, Na replaces K only when K_{ex} is <2 mM (Negendank and Shaller, 1979*a*), Na exchange in cells in >2 mM K_{ex} should have properties similar to S_1, whereas Na exchange in cells in <2 mM K_{ex} should have properties similar to S_2.

^{22}Na efflux was studied in cells pre-equilibrated in medium containing between 0 and 30 mM K_{ex}. Under these conditions, saturable fractions of K and Na are normal (133 and 11 mmol/kg cells wet wt, respectively) between 30 and 2 mM K_{ex}, half of K is replaced by Na at

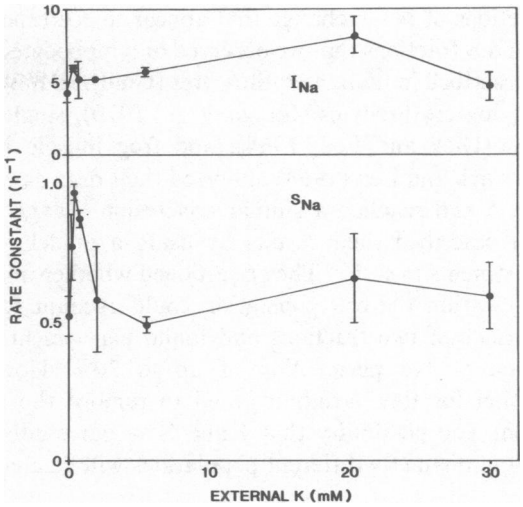

FIGURE 15 Na self-exchange in cells pre-equilibrated for 24–48 h in different concentrations of K_{ex} (where K_{ex} + Na_{ex} = 150 mM). Mean ± SEM, n = 3–8.

0.4 mM K_{ex}, and Na replaces K (7 mmol K and 129 mmol Na/kg cells wet wt) at 0 K_{ex} (Negendank and Shaller, 1979*a*, 1979*b*). The rate constants of the I_{Na} and S_{Na} fractions are summarized in Fig. 15. The results suggest: (*a*) K_{ex} has no consistent effect on the rate of Na exchange in I. (*b*) Above 2 mM K_{ex}, where the cellular content of Na remains normal, the rate of ^{22}Na exchange is independent of K_{ex}, and we assume that this is a property of Na in the S_1 fraction. (*c*) When Na replaces adsorbed K in cells incubated in <2 mM K_{ex}, its rate of self-exchange does vary with K_{ex}. The rate increased between 2 and 0.4 mM K_{ex}, and then decreased below 0.4 mM K_{ex}. We assume that this behavior is a property of Na in the S_2 fraction.

DISCUSSION

Discussion of the results is organized around the four questions raised at the end of the Introduction regarding: (a) the curvature of ^{22}Na efflux, (*b*) the properties of the I fraction of Na self-exchange, (*c*) the separation of S_1 and S_2 fractions of Na self-exchange, and (*d*) the nature of S_2 and its relation to K self-exchange.

Curvature of ^{22}Na Efflux

These studies suggest that the curvature of ^{22}Na efflux is not due to simple bulk-phase diffusion because of the following: (*a*) The diffusion coefficients (Fig. 5) were 5–7 orders of magnitude smaller than they were in dilute NaCl solution and could not possibly characterize ionic diffusion within cellular water. (*b*) The curve did not fit a simple diffusional pattern (Fig. 6). (*c*) The I and S fractions could be manipulated independently (Figs. 8 and 9). (*d*) The I and S fractions had different temperature dependences (Figs. 13 and 14).

Fractions of Na exchange that appear to correspond to the I and S fractions that we observed in lymphocytes have been described in human erythrocytes (Gold and Solomon, 1955), dog erythrocytes (Lange et al., 1970), single toad oocytes (Dick and Lea, 1964), and frog muscle (Ling, 1962). Dick and Lea (1964) analyzed their data as we did in Fig. 5 and reached a similar conclusion. Lange et al. (1967) described their results by using a model of two compartments in series. They questioned whether inhomogeneity within the cell population could account for the appearance of two fractions and found experimentally a variation of Na permeation of up to 30%. However, correction for this variation failed to remove the faster fraction. The possibility that I and S in our studies are present in distinctly different populations will be analyzed below.

Although not addressed in the studies reported here, the separate F fractions of Na and K were documented by (*a*) the rapid net gain or loss of this fraction of Na in media of varying NaCl concentration (Negendank and Shaller, 1981, 1982*c*), (*b*) the increase of the relative amount of Na in the F, compared with the I and S, fractions when preloading with isotope is brief (Negendank and Shaller, 1980*b*), and (*c*) the identity of the F fraction of K with the fraction of K that increases in a nonsaturable manner with an increase in K_{ex} (Negendank and Shaller, 1979*b*).

Properties of the I Fraction of Na

The amount of Na in the I fraction was about 10 mmol/kg cell or 20–30% of the normal amount of cellular Na. The amount did not increase when the cells gained Na, and the rate of Na self-exchange had little or no temperature dependence between O° and 37°C. These properties clearly differentiate the I fraction(s) from the slower, exponential S fraction. The Na in I was not chelated (chemically bound) because it exchanged readily with ^{22}Na ($t_{1/2}$ of 10–12 min).

To assess the conceivable source of I, one must consider the possibilities (*a*) that I has its origin on the cell surface, (*b*) that I and S are both intracellular, but located within different cells, and (*c*) that I and S are both located within the same cell. The Na in I could not simply be dissolved in a matrix covering part of the cell because its diffusion coefficient would have been about six orders of magnitude smaller than the coefficient for diffusion in the external aqueous solution (Fig. 5). Therefore, if the origin of Na is the cell surface, it would have to be bound to anionic sites. For a cell 10 μm in diameter, which has a convoluted surface area that is three times that of a sphere (Lichtman et al., 1972), there would have to be one Na bound for every 60–70 Å^2 of surface area (Negendank and Shaller, 1979*b*). It is not clear what the source of these sites would be. Based on studies of electrophoretic mobility, sialic acid residues appear to account for about half of the surface charge of human lymphocytes (Vassar et al., 1973), but this would not provide nearly enough sites. The maximum density of phospholipid head groups in a bilayer membrane is about one per 70 Å^2 (Small, 1967), but each group would have to be ionized and would have to bind Na in order to account for the I fraction of Na.

If I and S fractions existed within different cells, then from the analyses in Table II for a parallel model, cells containing I would occupy one-third or more of the total population of cells under control conditions. Exchanges of Na and K in these cells would be insensitive to metabolic inhibition, ouabain, and temperature. Therefore, assuming that all gained Na were confined to a subpopulation of cells containing S, then a gain of Na to as high as 139 mmol/kg cells (178 mM) (calculated on the basis of the entire cell population [Table I]) would mean that the cellular Na in that subpopulation would actually be much higher and, if freely dissolved, would make the cell hypertonic; yet the cells studied under these conditions did not swell (Table I). Moreover, because there is no I fraction of K exchange (Fig. 1), the subpopulation of cells containing I would have rates of K exchange one or two orders of magnitude slower. For these reasons, we suspect that I and S exist largely within the same cells.

If I and S exist within the same cells, then it is possible that I exists within the cytoplasm and normally exchanges in series with S, which exists within the nucleus. (The nucleus occupies some two-thirds of the volume of the lymphocyte.) However, when the cells gain Na it appears primarily in S_2, so S_2 must be found in a rather large compartment. If Na in S_2, or the K that it replaced, were in the nucleus and were dissolved freely in nuclear water, there would be a large osmotic gradient between the nucleus and the cytoplasm as we showed for K (Negendank and Shaller, 1979*b*). A second possibility is that the I fraction of Na is confined to membranous organelles (mitochondria, vesicles, etc.). A third possibility is that Na in I is adsorbed somewhere within the cytoplasm, the nucleus, or both.

Features of the S_1 and S_2 Na Fractions

S_1 contains the slowly exchanging Na that exists in the normal cell under control conditions (Figs. 4, 7–10) and in cells in >2 mM K_{ex} (Fig. 15). Its rate is independent of temperature over the range in which it can be determined (37°–15°C) (Fig. 14, left side), and its amount and rate are independent of K_{ex} at K_{ex} between 2 and 30 mM (Fig. 15).

S_2 appears when the cells replace K with Na. Its separation from S_1 is shown by its steep temperature dependence in cells in 5.4 mM K_{ex} only at temperatures (<15°C) at which Na is gained (Fig. 14, left) and by its steep temperature dependence over the range of 0–37°C when cells gain Na when bathed in ouabain (Negendank and Shaller, 1982*c*) or in low K_{ex} (Fig. 14, right). The rate of Na self-exchange in S_2 varied according to the means by which the cells were induced to gain Na: it was fast when

ATP was depleted (Fig. 8 *b*), slow (and about the same as in S_1) in ouabain (Fig. 8 *a*) or in low K_{ex} (Fig. 15), and very slow at 0°C (Figs. 9, 13, 14). Its rate varied markedly in cells that were equilibrated in K_{ex} between 0 and 2 mM (Fig. 15).

In the conventional approach to cellular physiology, it is assumed that processes within the surface membrane control cellular ionic contents and exchanges. It is assumed that the normal net exclusion of Na is maintained by a pump whereas the normal net accumulation of K is a result of a Donnan equilibrium, a pump, or both. In this theory, it is mandatory that the slow exponential K exchange (Fig. 1) and the slower exponential fraction of Na exchange (Figs. 2, 4 and 7) are caused by processes that are limited to the surface membrane and subject to the effects of ouabain (Fig. 8 *a*), metabolic inhibition (Fig. 8 *b*), temperature (Figs. 9, 11–13), and altered K_{ex} (Figs. 10, 15). It is also mandatory that the F and I fractions of ^{22}Na exchange either be external to the plasma membrane or present within subpopulations of cells that are not sensitive to the above-mentioned effects and do not replace K with Na.

Results of prior studies in lymphocytes contradict the membrane theory (Negendank, 1982). Among the many reasons for this are (*a*) an inability to explain the F fractions of exchange of K and Na by an extracellular or cell surface source (Negendank and Shaller, 1979*b*, 1980*b*) and (*b*) failure of the Na efflux to slow, which would have accounted for the net gain of Na that was postulated to have been caused by a decrease in the rate of outward-directed pumping of Na (Negendank and Shaller, 1982*a*, 1982*c*). The first problem is confounded by the need to explain the I Na fraction. The second problem is also underscored by the results presented here. The net gain of Na was not accompanied by a decrease in Na efflux when the cells were treated with ouabain (Fig. 8 *a*), depleted of ATP, incubated at 0°C (Fig. 9) or incubated in 0 mM K_{ex} (Fig. 15). Moreover according to the membrane theory, in control cells Na exchange in S_1 must be caused by the normal outward-directed pumping of Na; yet, the slight temperature dependence of S_1 between 37° and 15°C (Fig. 14, left) is not characteristic of an enzymatic process and does not match the marked temperature dependence of the putative Na, K-ATPase pump, which has been isolated from human lymphocytes (Negendank and Shaller, 1982*d*). At the same time, S_2, which appears in cells in which the apparent Na gradient is completely dissipated, should mirror passive membrane processes; yet, it has a steep temperature dependence (Negendank and Shaller, 1982*c* and Fig. 14, right).

Nature of the S_2 Fraction of Na

From earlier studies we concluded that the slow exponential fractions of exchange of K and Na are intracellular in origin and limited by interaction with fixed charges on intracellular macromolecules whereas the F fractions are caused by the permeability of the surface membrane (Negendank and Shaller, 1979*b*, 1980*b*). In the theory that we have adopted (Ling, 1962, 1984), three distinct sets of ion exchange or diffusional processes occur. One is the exchange between ions in the extracellular medium and ions dissolved within the cellular water. This process is limited by the permeability of the surface membrane. The second is the diffusion of ions within cellular water. Although in this process cellular water is ordered, or polarized, in multilayers, the diffusion coefficient of these ions is expected to be only slightly less than that in ordinary water (Negendank, 1982). The diffusion of ions in cell water, which is likely to occur with a diffusion coefficient on the order of 10^{-6}–10^{-5} cm^2/s, is very fast and was not detected in our experiments. Hence, the $t_{1/2}$'s of 2–4 min of the F fractions of cellular exchanges of K and Na, which would correspond to a diffusion coefficient on the order of 10^{-10} cm^2/s (Fig. 5), are caused by the slow penetration of these ions through the surface membrane. The third ionic self-exchange process is the exchange between ions in cellular water and ions adsorbed onto intracellular macromolecular fixed charges. This process is manifested by the self-exchanges of Na in S_1 and S_2 (and possibly in I) and of K in its S fraction.

Within this context, the general properties of the fractions of self-exchanges of Na and K are summarized in a schematic diagram of the human lymphocyte shown in Fig. 16. The results presented here suggest that Na exchange in S_2 and K exchange in its S fraction have a similar interaction with the same fixed-charge sites. These sites are shown in Fig. 16 as a larger zig-zag line, and are the ones that are influenced by ATP, temperature, and ouabain.

Independent physical evidence for adsorption of monovalent ions (especially K) within cells was reviewed briefly

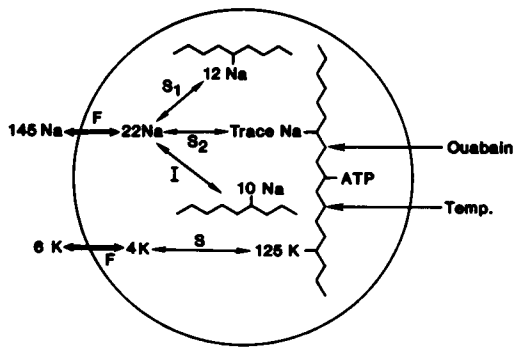

FIGURE 16 Schematic model of the lymphocyte under control conditions at 37°C. Ionic concentrations in the external medium and in cellular water are expressed in millimoles per liter; concentrations of adsorbed ions are expressed as millimoles per kilogram wet cell weight. The large set of ion-adsorbing sites normally contain K, and when K is lost (in low K_{ex}, in ouabain, at low temperature, or when ATP is depleted), it is replaced by Na; this Na then exchanges in the S_2 fraction. The sources of the S_1 and I fractions could be adsorption sites, as shown, or compartments within subcellular organelles.

(Negendank, 1982) and in depth in a recent book (Ling, 1984). We do not know what the K-adsorbing proteins are in lymphocytes, but it is clear that the ion-adsorbing sites behave in a relatively uniform fashion, as deduced from the evidence that demonstrates that there is a cooperative interaction between neighboring sites. This evidence includes the steep, sigmoidal equilibrium distribution isotherms of K and Na in which half of the sites adsorb K and half adsorb Na at 0.4 mM K_{ex} (Negendank and Shaller, 1979*a*); the critical temperature transition of Na/K exchange, which occurs over the narrow range 0°–10°C with 50% exchange occurring at 3°C (Negendank and Shaller, 1980*a*); and the allosteric nature of the effect of ouabain on the K and Na equilibrium distribution isotherms (Negendank and Collier, 1976). Moreover, the kinetics of the net replacement of Na by K in lymphocytes are determined by the K-induced autocooperative transition in the relative affinities of the sites for K and Na, and the data fit theoretical expectations based on a stochastic treatment and on a hydrodynamic solution of the time-dependent Ising model (Negendank and Karreman, 1979; Huang and Negendank, 1980). In this context, we suggest that the peaks in the rates of ionic self-exchanges (S_{Na} in Fig. 15 and S_K in Negendank and Shaller, 1979*b*), which occur near the cooperative transition at 0.4 mM K_{ex}, are somehow related to the cooperative interaction between these sites. However, investigation of this phenomenon requires further study.

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